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# Identification of cDNAs induced by the organophosphate trichlorphon in the parasitic copepod *Lepeophtheirus salmonis* (Copepoda; Caligidae)

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**Abstract**

*Lepeophtheirus salmonis* is a copepod ectoparasite of wild and farmed salmonids and can cause considerable damage to infected fish. We have examined the effect of the organophosphate trichlorphon, which was one of the early treatments for sea lice as Neguvon® on *L. salmonis*. Suppression subtractive hybridisation was used to prepare a cDNA library enriched for cDNAs expressed in response to the organophosphate trichlorophon and using this technique a total of 33 cDNAs were found to be differentially regulated in response to organophosphate exposure. Sequence analysis of the cDNAs revealed that many were involved in cellular stress responses and or neurotoxicity. The expression of two of these cDNAs was confirmed to be up-regulated in response to trichlorophon.
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## 1. Introduction

*Lepeophtheirus salmonis* (Krøyer) or sea louse is a copepod ectoparasite of wild and farmed salmonids [1]. Since the large-scale marine farming of Atlantic salmon (*Salmo salar*) began in the 1970s, *L. salmonis* has been recognised as a major commercial and animal welfare problem [2,3]. Severe infestation can lead to high mortality but even moderate lice numbers can affect the condition and therefore the growth rate of salmon [4]. As one of the few economically important copepod species, *L. salmonis* has attracted more research than most but in general little is known about the biochemistry of copepods.

Treatments for sea lice have generally been developed from insecticides and there have been some reports of resistance, particularly to organophosphates and pyrethroids [5–7]. A number of different treatments have been used but many have been discarded due to poor efficacy, cost, difficulties in application, environmental problems or regulatory difficulties [8]. The limited number of treatments available and the demonstrated ability of sea lice to develop resistance makes it vital that we understand how these organisms deal with the chemotherepeutants used to treat sea lice infections. This work focuses on identifying some of the proteins involved in the response to chemotherepeutants using the organophosphate trichlorphon which was one of the early treatments for sea lice as Neguvon® [1].

Organophosphates are potent neurotoxins and their toxicity is caused by blocking the breakdown of acetylcholine by acetylcholine esterase (AChE) [9]. Inhibition of AChE results in a build up of acetylcholine causing continuous and excessive stimulation of the nerve and muscle fibres leading to spastic paralysis and death [10]. While organophosphates are no longer used to treat sea louse infestations in Scotland due to the development of resistance, the use of emmamectin benzoate and wider environmental impacts, organophosphates such as dichlorvos, a metabolite, and the major toxic derivative of trichlorphon [11], are still widely used to control external copepod parasites in aquaculture elsewhere in the world [12,13].

In this work, a number of cDNAs have been identified by suppression subtractive hybridisation (SSH) as encoding

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for proteins that are potentially involved in the stress and/or detoxification response of *L. salmonis* towards the organophosphate trichlorphon.

## 2. Materials and methods

### 2.1. Sea lice treatment

Adult female *L. salmonis* were collected at harvest from adult Atlantic salmon in Loch Eil on the west coast of Scotland. Adult female *L. salmonis* were selected for treatment if they were responsive to stimulation with forceps after acclimatisation in a controlled temperature room (10 °C) overnight. Treatment was carried out at 10 °C in 2 ml of autoclaved sea water to which trichlorphon was added in 32-well plates. A 100 ppm stock solution of trichlorphon was made up in 95% ethanol immediately prior to dilution and treatment. Three replica plates with lice at two concentrations of trichlorphon, 1.0 and 0.5 ppm were prepared. Controls were also prepared and contained an equivalent amount of ethanol as the treated samples. Adult females were individually transferred to the treatment wells with forceps and exposed for one hour. They were then transferred to fresh filtered autoclaved seawater and allowed to recover for 2 h, prior to being frozen in liquid nitrogen and stored at −70 °C.

### 2.2. RNA manipulation

PolyA$^+$ RNA extraction and purification was performed from frozen samples with an Oligotex® Direct mRNA Mini Kit (Qiagen) and samples were manually disrupted using a pellet pestle in the kits extraction buffer. Reverse transcription to cDNA was performed using the CLONTECH SMART™ PCR cDNA Synthesis Kit which enables proportional amplification of full length cDNAs.

### 2.3. Library construction

Suppression subtractive hybridisation was performed using the CLONTECH PCR-Select™ cDNA Subtraction Kit (BD Biosciences) [14,15]. The efficiency of the subtraction was checked by PCR amplification of subtracted and unsubtracted cDNA using β-actin primers. Subtracted cDNA was then cloned into the pGem-T Easy vector (Promega), transformed into *Escherichia coli* (XL1 blue) and plated out onto LB plus ampicilin, IPTG and XGal agar plates and grown overnight at 37 °C. White colonies were picked into 96-well plates and incubated overnight in LB plus ampicilin at 37 °C. Replica plates were made and to one, glycerol 15% (v/v) was added and stored at −70 °C, working plates were stored at 4 °C.

### 2.4. Screening and identification of clones

Screening of the subtracted cDNA was performed by amplifying the clones by PCR with vector specific primers flanking the multiple cloning site, (195) and (196), and those plasmids containing inserts greater than 200 bp in length were selected for further screening. Equal amounts of DNA were spotted onto nylon membranes which were then probed with DIG labelled treated and untreated subtracted cDNA, hybridisation was detected by chemiluminescence according to the manufacturers instructions (Roche).

Plasmid DNA from the positive hybridising clones was prepared using a Qiagen Mini-Prep Kit and sequencing was performed using the vector specific primers. Sequence data was analysed using the National Centre for Biotechnology Information (NCBI) BLASTx program [16].

Table 1
Oligonucleotide primers used

| No. | Sequence 5′–3′ | Template |
|---|---|---|
| 195 | GGAAACAGCTATG ACCATG | Forward pGEM-T |
| 196 | GTTTTCCCAGTCACGAC | Reverse pGEM-T |
| 356 | CACTTGCCCGAAGAAGTTCCA | Forward PDI |
| 357 | GTAGTGACAACTTCGTTACTTACCTTC | Reverse PDI |
| 358 | GGGACTACACGTCTGAGTTCC | Forward CRABP |
| 359 | TCTCCTCGACTTCTTTCCGAC | Reverse CRABP |
| 330 | GCCTCTGGTCGGACCACTGGGATT | Forward actin |
| 331 | CCATCCCAAGAAAGAGGGTTGG | Reverse actin |

### 2.5. Real-time PCR

Real-time PCR [17] was used to examine the expression of some identified mRNAs in response to exposure to trichlorphon by relative quantification of the mRNA (as cDNA). Transcripts were quantified by real-time reverse transcription PCR (RT-PCR) using the LightCycler (Roche) and SYBR® Green I master mix according to the manufacturers instructions (Roche). Quantification was based on amplicons of less than 400 bp using internal primers (Table 1). Real time RT-PCR experiments were replicated at least three times and the amount of cDNA present normalised against expression of the house-keeping gene β-actin, which was measured in parallel PCRs using actin specific primers (Table 1).

## 3. Results and discussion

In view of the fact that organophosphates were one of the early treatments used against *L. salmonis* we sought to identify genes whose expression is induced as a result of organophosphate exposure. Adult female *L. salmonis* were collected from a fish farm and exposed to a number of different concentrations of the organophosphate trichlorphon, the active component of Neguvon®, and the effect on the lice examined. The results showed that there was initial paralysis in 50% of the lice exposed to 0.5 ppm trichlorphon and in 75% of lice treated with 1.0 ppm trichlorphon. Paralysed lice were observed to float to the surface, while the control and the unaffected lice remained attached to the sides or the bottom of the treatment vessel. Moreover, the affected lice did not respond when moved into untreated seawater to recover, while sea lice in the control and those

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